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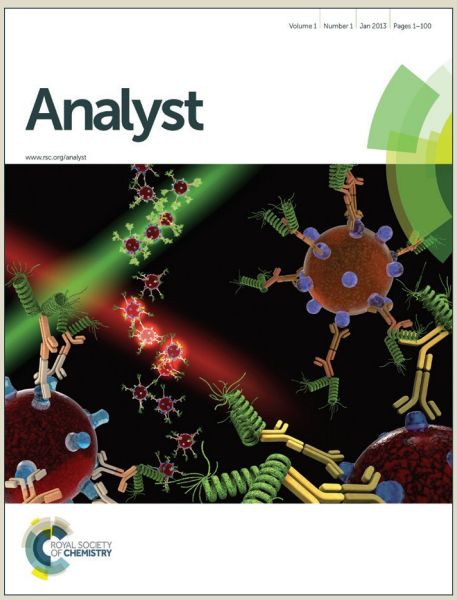

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ARTICLE

Affinity imaging mass spectrometry (AIMS): high-throughput screening for specific small molecule interactions with frozen tissue sections

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A novel screening system, using affinity imaging mass spectrometry (AIMS), has been developed to identify protein aggregates or organ structures in unfixed human tissue. Frozen tissue sections are positioned on small (millimetre-scale) stainless steel chips and incubated with an extensive library of small molecules. Candidate molecules showing specific affinity for the tissue section are identified by imaging mass spectrometry (IMS). As an example application, we screened over a thousand compounds against Alzheimer's disease (AD) brain tissue and identified several compounds with high affinity for AD brain sections containing tau deposits compared to age-matched controls. It should also be possible to use AIMS to isolate chemical compounds with affinity for tissue structures or components that have been extensively modified by events such as oxidation, phosphorylation, acetylation, aggregation, racemization or truncation, for example, due to aging. It may also be applicable to biomarker screening programs.

Introduction

There is a widely recognized need to image pathogenic proteins for diagnosis and therapy, especially for the early detection of diseases such as cancer (1), inflammation (2) and neurodegenerative disorders (3). Several approaches have been used to identify chemical compounds or drugs for imaging applications in diagnosis or therapy, for example involving biosynthesis (4) or distribution in mice (5). However, in vitro or cell-based experiments cannot fully reflect the state of pathogenic organs, in which organ structures are affected by modifications, such as oxidation, phosphorylation, acetylation, truncation, aggregation and glycosylation (6, 7), and screening approaches using the affected tissues directly would be preferable.

Matrix-associated laser desorption/ionization mass spectrometry (MALDI-MS) has been used extensively for the analysis of relatively pure samples, e.g. purified peptides or chemical compounds. The recent development of extremely precise, high-resolution systems has allowed MALDI-MS to be increasingly used for the direct detection of molecules in tissue samples (8). For example, imaging mass spectrometry (IMS) is a mapping method based on mass spectrometry that visualizes the distribution of drugs or biological molecules in tissue sections (9, 10). Images are reconstructed from the mass spectra of the thousands of spots obtained by raster analysis of the biological tissue. IMS can analyze thousands of molecules simultaneously from one point of ionization, and subsequently allows the selection of a single molecule to map its distribution in the tissue. This ability to analyze large numbers of compounds simultaneously makes IMS highly suitable for drug screening. However, while IMS has proved useful for determining the distribution of a drug candidate and its metabolites in a tissue sample, and also for pharmacokinetics (11, 12, 13), a high-throughput approach, whereby IMS is used to screen millions of drug candidates against thousands of tissue sections, has not been described.

We have developed a novel IMS-based small-molecule screening system using frozen human tissue, which faithfully reflects any associated pathogenic features and allows selection of compounds with affinity for specific components or structures in the tissue. The screening system has two steps: (a) attachment of ultrathin frozen sections to individual stainless steel microchips, which are then incubated in solutions of a library of chemical compounds; (b) detection of compound binding to the tissue sections by IMS. This system, which we call AIMS (affinity IMS), allows the identification of

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compounds that can be used to image any specific structure or molecule in the unfixed frozen section. Here we present the results of high-throughput screening for molecules with affinity for tau deposits in human brain and identify 14 compounds out of thousands in a small molecule (<500 MW) library.

Results and discussion

Affinity imaging mass spectrometry: a novel high-throughput approach to small molecule screening

We have developed a novel screening system to identify chemical compounds with affinity for any organ, structure, or protein in a tissue. This system uses stainless steel microchips to hold multiple millimetre-scale frozen brain sections that are then incubated in solutions of library compounds. After washing, those compounds that have affinity for the organ structure are visualized by the imaging mass spectrometry method.

The microchips are manipulated by a robot hand system, as shown in Figure 1, which enables us to make 16,000 sections from a 1 cm^3 cube of organ sample and thus to identify affinity chemicals even for rare targets. After a frozen tissue section is laid on the 4 x 4 array of microchips in the holding tray (Fig. 2), each chip is pushed out from beneath by a pin (Fig. 1); this allows recovery of individual chips from the tray and simultaneously divides the frozen section into fragments attached to each chip. Subsequently, chips are transferred to a custom-made 96-well plate using a robot hand (DENSO VP-G2 6-axis robot for medical use). The chuck of the robot hand is specially designed to grip the chip and the tray. Each microchip of a 'chip set', defined as an AD brain section paired with a control section, is placed side by side in the 96-well plate and immersed in the same diluted chemical solution. The compound library used here is the 'core library' of 9600 compounds provided by Drug Discovery Initiative, University of Tokyo. After incubation with the individual compounds of the library, each chip set is transferred to another custom-made tray for IMS detection, as shown in Fig. 3a. A 2 x 1 mm area extending across part of both the AD and control sections (Fig. 3b) is analysed by IMS, with a representative image shown in Fig. 3c.

Screening for compounds with affinity for AD brain sections with tau deposits

To demonstrate the potential of the AIMS system, we screened 3200 chemical compounds from the library against brain tissue containing tau deposits (Fig. S3). We obtained 14 positive compounds, which showed a higher intensity of the relevant ion in the AD brain sections compared to the control brain region of the chip set; an example is shown in Fig. 4. The relative affinity for the AD brain region was evaluated as a tau-positive score given by the ratio of the mean intensity of the AD area divided by that of the control area, after subtraction of the intensity value of the negative control chip set. For a compound as shown in Fig. 4, the tau-positive score is calculated as (16/7)/(7/6)=1.96. The score (approximately 2) means that the compound has a fairly good affinity to the tau-deposited brain. The negative control chip set was treated in the same way as the experimental chip sets, but was not exposed to any library compounds. Two further examples of positive compounds are shown in Fig. S4. Screening was performed at least three times in separate experiments and candidates were selected if the tau-positive score was >2 on at least two occasions, resulting in 25 compounds with affinity for AD brain sections. Following MS/MS analysis, 14 of the 25 compounds were shown to have a fragment ion relevant to its precursor (parental ion). These compounds have variable structures, which cannot be disclosed at this point due to intellectual property considerations.

Nanoparticles as an alternative matrix for MALDI-TOF

One drawback of the AIMS system is the unpredictable ionization efficiency of each target compound, which can be an issue with any ionization technique. To overcome this, we used nanoparticles as an alternative matrix, which we termed a 'nanomatrix', for IMS (14). The nanomatrix provides a high signal-to-noise ratio, especially for low molecular weight (MW 100-500) compounds, independently of their chemical structure. As a result, more than 60% of the library compounds, as detected by both positive and negative modes, could be ionized (Table 1).

Detection specificity of the imaging system

To confirm the specificity of images obtained with this system, we performed MS/MS analysis of a compound, PBB3, known for its tau-specific binding characteristics (15; Fig. 5). Comparing the images derived from fragment ions (m/z 282 and 202; Fig. S1) with that of the parental ion (m/z 310), we observed multiple overlapping dots, as indicated by arrows in the merged image.

Detection of multiple compounds by AIMS

The AIMS system also allows us to analyze multiple chemical compounds on a single chip set simultaneously. For example, when solutions of eight compounds (3 µM each), mixed in 50% ethanol, were used for chip set incubations, we detected seven out of eight by AIMS (data not shown). The detected ion intensities on the image (either AD or normal control) were reproducible.

Further applications

The AIMS system enables the screening of novel chemical compounds with specificity for the complicated human pathologies that accompany aging or disease, including pre-symptomic states. It can be used to screen any structure in the human body greater than 35 µm in size using the AXIMA

Resonance mass spectrometer. If apparatus with a higher spatial resolution, e.g. the iMScope (Shimadzu Co. Japan), is available, it could be used to detect targets smaller than 10 µm, including single cells within tissues. This new technology should be invaluable for the development of PET probes for diagnosis of neurodegenerative diseases such as AD, Parkinson's disease, dementia with Lewy bodies, Huntington's disease, amyotrophic lateral sclerosis, and frontotemporal dementia. It is envisaged that it will also potentiate the development of biomarkers for the detection of early stage cancer, and may be used to validate drug delivery.

Conclusions

We introduce a novel screening system, which uses affinity imaging mass spectrometry (AIMS) to identify chemical probes for the visualization of specific molecular events, such as protein aggregation, in target tissues. Such probes could be used for therapeutic assessment (e.g. by PET) and for validation of disease biomarkers, and may also have applications in drug discovery.

Experimental section

Human brain samples and immunohistochemistry

An Alzheimer's disease frontal cortex sample, which shows extensive tau deposition, and an age-matched normal frontal cortex (control) were provided by Brain Bank for Aging Research, Research Team for Geriatric Pathology, Tokyo Metropolitan Institute of Gerontology. The AD sample contains neurons that stain positively using antibody AT8 (16) for phosphorylated tau (P-tau) across the whole area; neurofibrillary tangles (NFTs) are also stained (Fig. S3a, d). The normal control frontal cortex does not stain with the P-tau antibody. Amyloid beta (Aß) deposition in both samples was detected using 6E10 antibody (Covance, No. SIG-39300), as shown in Fig. S3b, e. A control experiment for both samples without primary antibody is shown in Fig. S3c, f. After Sudan Black staining for 1 h, indirect immunohistochemistry was performed by incubation with primary antibody (1/100 dilution for AT8; or 1/200 dilution for 6E10) at room temperature (RT) for 1 h, followed by visualization using an Alexa Fluor 568-conjugated anti-mouse secondary antibody (1/400 dilution). Photographs were taken with a fluorescent microscope with WIG filter (BX50/DP70 system microscope, Olympus Co. Tokyo, Japan).

Microchip system for frozen sections

Square microchips (W 2.4 mm, D 2.4 mm, H 1.0 mm) were wire cut from a 1 mm-thick SUS304 stainless steel plate (Fig. 2a,b). The holding tray (Fig. 2c) was made to hold 16 microchips in a 4 x 4 array, which can be overlaid with a frozen tissue section (10 mm x 10 mm, 12 µm thick). The tray has 16 holes (each 2 mm diameter), one beneath each chip section, to allow chips to be pushed out by a separator (Fig. 3, insert) and then to be transferred to a custom-made 96-well plate (described below) using a robot hand system (DENSO VP-G2 6-axis robot). The chuck of the robot hand is specially designed for gripping both microchips and the tray. One microchip with an AD brain section attached and one with a control section (together termed a 'chip set') were placed in adjacent wells in the plate for treatment with the same diluted chemical solution.

Custom-made 96-well plate for solution treatment of microchips

A 96-well plate, designed to hold one 2.4 mm-square microchip in each well, was custom-made using a metal mold (Fig. S5). The design enables the microchips to be handled easily by the robot hand system; a special plastic (Microresico®, Richel Co. Toyama, Japan) is used to make the plate to ensure minimal compound adsorption to surfaces. Two pegs are located on both sides of the wall of each well to hold the microchip at a medium depth in the well. The bottom of the well is sloped to allow easy removal of chemical solutions.

Chemical compound library

To screen for compounds that specifically interact with AD brain samples, we used the 'core library' (9600 compounds) for drug discovery provided by the Drug Discovery Initiative, University of Tokyo, Pharmaceutical Science Main Building, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan (http://www.ddi.u-tokyo.ac.jp/en/#4). The compounds, which were supplied as 10 mM solutions in DMSO, were first diluted to 100 µM in 50% acetonitrile, 0.1% trifluoroacetic acid using a Laboratory Automation System (Beckman Co., Biomek NX^P and FX^P) and stored at -20ºC using a 2D barcode storage system (STT1000 Kiwi, Liconic Instruments, Liechtenstein and Boston). For imaging mass spectrometry (see below), the 100 µM solutions were further diluted 1/10 using 50% ethanol for incubation with microchips. In this study, we have totally analized 9520 compounds from the core library.

Selection of chemical compounds for imaging mass spectrometry

To collect the m/z values of parental ions, 0.8 µL of nanomatrix (see below) was spotted onto a standard target plate, after which 0.8 µL of each 100 µM solution was spotted onto the nanomatrix spots. Then spectra were recorded after accumulation of data from 121 individual spots (two laser shots per spot) using an AXIMA Resonance time-of-flight mass spectrometer (Shimadzu Corporation, Kyoto, Japan). Chemical compounds that showed intense molecular ions (e.g. [M]+/[M+H]+/[M+Na]+/[M+K]+ in positive mode or [M]-/[M-H]- in negative mode) were selected for further MS-imaging. Intensity values of 12000 (in positive mode) or 4000 (in negative mode) were set as threshold. The specificity was confirmed by ion trap/time-of-flight MS/MS analysis on the apparatus.

Nanomatrix for imaging mass spectrometry

Nanoparticles, prepared as reported previously (14), were used as assistant matrix for IMS. Briefly, 100 mL 2% $FeCl_2 \cdot 4H_2O$ solution was mixed with 100 mL 3-aminopropyltriethoxysilane (γ -APTES; Shin-etsu Kagaku, Japan) by vigorous stirring at RT. After cooling for 1 h and centrifugation at 12000g for 20 min at 4ºC, the precipitate was sonicated for 5-10 min in ultrapure water and subsequently washed several times by centrifugation in ultrapure water and once in ethanol. The precipitate was dried at 45ºC for 3 h. The dried sample (10 mg) was pulverized in a pellet mixer, and sonicated six times in a microcentrifuge tube with 500 µL methanol or acetonitrile. After centrifugation at 10000g for 10 s, the supernatant (0.8 µL) was used for the precursor ion analysis and for imaging mass spectrometry after 1/10 dilution. The nanomatrix was hand-sprayed on the microchips with sections on the holding tray using an air brush system (PS270 and PS313, GSI Creos, Tokyo, Japan). In the case of MS/MS imaging analysis, 2,5-dihydroxybenzoic acid (DHB; 50 mg/ml in 99.5% MeOH) was used as matrix.

Preparation of the small frozen section attached to the microchip

The frontal cortex regions of the AD and the control brain were provided as a 7 mm-thick coronal block. Sample blocks approximately 14 mm-square and 7 mm-thick were prepared with an ultrasonic cutter (SUW-30, Suzuki Co., Japan) and the sample was mounted in a cryostat sample holder (CM1950, Leica Microsystems, Nussloch, Germany) using a minimal amount of optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Japan). Cut sections (12 µm thick) were placed on the tray containing 16 microchips, then each microchip was warmed from the underside by finger contact to attach the section to the chip. The tray was incubated at 37ºC for 1 h to dry and to immobilize the tissue section, after which it was stored at 4ºC if used within two weeks, or otherwise stored at -20ºC. Using the robot hand and separator system, each chip set (microsections of AD and control sample) was transferred to the custom-made 96-well plate, with the paired microchips placed in adjacent wells.

Immersion in chemical compounds

Each chip set was soaked once in 50% ethanol for 2 min, then subsequently immersed in 10 µM chemical solution (55 µL per well) as described above for 1 h, washed once in 50% ethanol, and dried for 1 h at RT. The immersion conditions were as used in an established method for staining tau in pathological specimens using Congo red, thioflavin and various derivatives (17). To validate the procedure, we used the tau-binding compound PBB3 (15) as a positive control as shown in Fig. 5. When multiple chemical compounds were combined, 3 µM of each of eight solutions were mixed in 50% ethanol prior to adding to the well. After soaking in either single or multiple compounds, the chip set was transferred back to the holding tray, with AD and control brain microchips placed side by side for IMS.

Imaging mass spectrometry

IMS analysis was performed on 2 x 1 mm regions spanning part of the microsections of both AD and control samples (Fig. 3b) on the custom target plate (Fig. 3a) with 35 µm raster step (58 x 29 = 1682 dots). Using the 'intensity mapping' feature, an MS-image was constructed for the ions of interest (e.g. molecular ion or specific fragment ions) and negative control images as shown in Fig. 4. Setting of the AXIMA Resonance used in the AIMS was thus; positive ion mode, laser power 65 which is an arbitrary units with a maximum of 180, laser repetition rate 5 Hz, accumulated 2 shots for a raster dot, and images are generated from intensities of parent ions (±0.05Da) using the Shimadzu AXIMA built-in software (intensity mapping). Alternatively, some data were exported and analysed with BioMap software (http://www.maldi-msi.org/). Exceptionally, the MS/MS imaging analysis (Fig. 5) was performed on 3 x 1.5 mm regions with 50 µm raster step (60 x 30 = 1800 dots), laser power 80, repetition rate 10 Hz, with 10 shots for a raster dot and MS/MS collision-induced dissociation (CID) power with 195 (resolution >500).

Human welfare

All experiments were performed in compliance with the relevant laws and institutional guidelines and approved by ethical committees of National Center for Geriatrics and Gerontology (authorization Nos. 609 and 609-2) and Tokyo Metropolitan Institute of Gerontology (authorization No. 24-1642-76).

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Table 1. Detection efficiency of precursor ions

Mode	Positive	Negative	Total
Detected	5119	652	5771
%	53.8	6.8	60.6

The precursor ions were detected over 60% efficiency out of 9520 compounds by the nanoparticle-associated mass spectrometry subjected to both positive and negative ion modes.

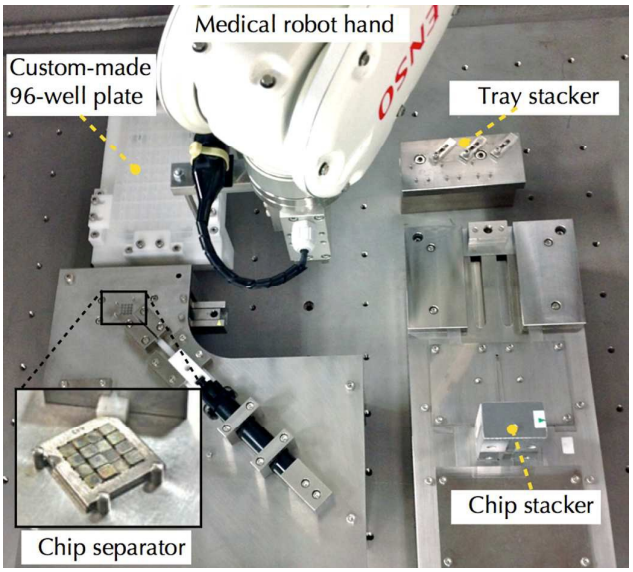

Fig. 1 The robot system. The robot hand was used to transfer microchips according to the following three steps. 1. From the chip stacker to the holding tray on the tray stacker. 2. From chip separator to 96-well plate. 3. From 96-well plate to the tray stacker after immersion in chemical compounds.

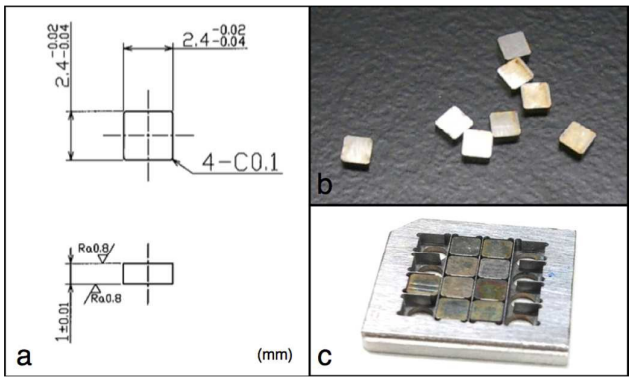

Fig. 2 Microchips and the holding tray. a. Design of the microchip. b. Examples of microchips. c. The holding tray accommodates 16 chips on which a frozen tissue section is overlaid.

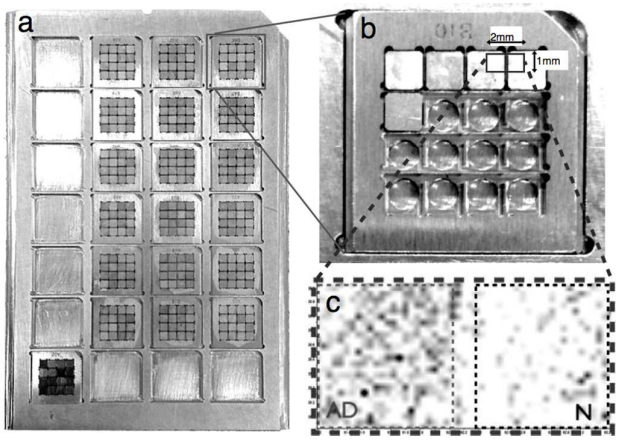

Fig. 3 Custom-made target plates for imaging mass spectrometry of microchips. The target plate (a) holds a 4 x 7 array of holding trays (b) each containing 16 microchips. A 2 x 1 mm area spanning both AD and control sections is analyzed by 58 x 29 dots with 35 µm raster step. In (c) is shown an example of preferential binding of a compound to the AD brain section (left side of panel).

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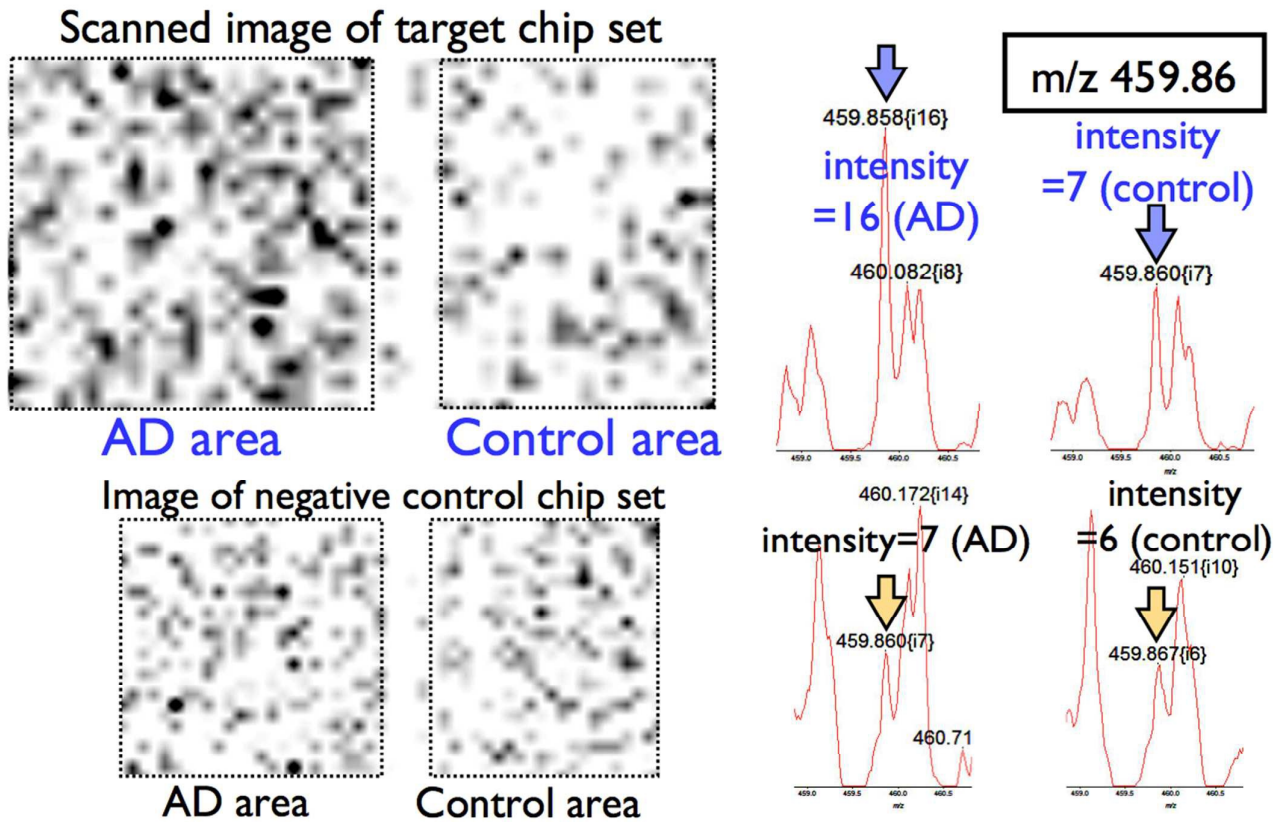

Fig. 4 Evaluation of compound binding by imaging mass spectrometry. The image was obtained by scanning a 2 x 1 mm region spanning the chip set of AD and control sections, with data accumulated at m/z 459.858 ±0.05. The corresponding mean intensity of the detected parental ions in both areas is shown to the right of the figure. The affinity for the tau-deposited region was evaluated as the ratio of the mean intensity of the AD area divided by that of control area after subtraction of the intensity value of the negative control section.

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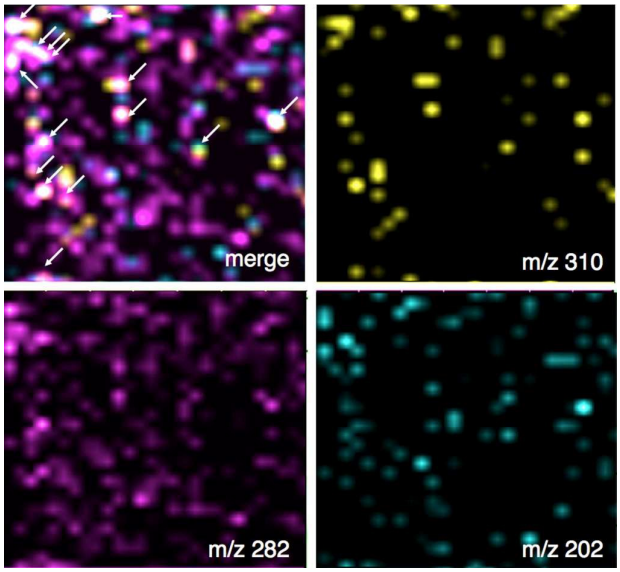

Fig. 5 Ion trap/time-of-flight MS/MS imaging of the tau-specific compound PBB3. The white arrows in the merged image show overlap detection of the parental ion (m/z 310) and the daughter ions (m/z 282 and 202). The image data were obtained by scanning a 1.5 x 1.5 mm region of the AD section. The mass spectra are provided as Figs. S1 and S2.